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Mutations in the Bacterial Ribosomal Protein L3 and Their Association with Antibiotic Resistance

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Different groups of antibiotics bind to the peptidyl transferase center (PTC) in the large subunit of the bacterial ribosome. Resistance to these groups of antibiotics has often been linked with mutations or methylations of the 23S rRNA. In recent years, there has been a rise in the number of studies where mutations have been found in the ribosomal protein L3 in bacterial strains resistant to PTC-targeting antibiotics but there is often no evidence that these mutations actually confer antibiotic resistance. In this study, a plasmid exchange system was used to replace plasmid-carried wild-type genes with mutated L3 genes in a chromosomal L3 deletion strain. In this way, the essential L3 gene is available for the bacteria while allowing replacement of the wild type with mutated L3 genes. This enables investigation of the effect of single mutations in *Escherichia coli* without a wild-type L3 background. Ten plasmid-carried mutated L3 genes were constructed, and their effect on growth and antibiotic susceptibility was investigated. Additionally, computational modeling of the impact of L3 mutations in *E. coli* was used to assess changes in 50S structure and antibiotic binding. All mutations are placed in the loops of L3 near the PTC. Growth data show that 9 of the 10 mutations were well accepted in *E. coli*, although some of them came with a fitness cost. Only one of the mutants exhibited reduced susceptibility to linezolid, while five exhibited reduced susceptibility to tiamulin.

During the last 10 years, mutations in the L3 ribosomal protein have been associated with bacterial resistance to linezolid (an oxazolidinone) and tiamulin (a pleuromutilin). These drugs bind to the ribosomal peptidyl transferase center (PTC), where specific 23S rRNA mutations and methylation at 2503 also cause resistance to oxazolidinones and pleuromutilins (reviewed in references 1, 2, and 3). The main part of L3 is positioned on the surface of the 50S ribosomal subunit, but a branched loop extends close to the PTC (Fig. 1), the binding site for many different ribosomal antibiotics. The first L3 resistance mutation in bacteria was detected in 2003 in *Escherichia coli* selected with tiamulin, and its role in resistance was verified by genetic evidence (4). Since then, L3 mutations have been associated with resistance to linezolid, tiamulin or valnemulin, and anisomycin, as reviewed in reference 1. L3 mutations related to antibiotic resistance have been reported in *Brachyspira* spp., *Staphylococcus* spp., *E. coli*, and *Mycobacterium tuberculosis*. Most of the studies have lacked proof that these mutations actually cause antibiotic resistance, except a few (4–7). It is not evident exactly which L3 mutations have a relation to reduced antibiotic susceptibility, but only those in the part of the L3 protein nearest to the PTC are assumed to confer a resistance effect. Most of the known L3 mutations are found together with other resistance determinants, mainly mutations in the domain V region of 23S rRNA or mutations in ribosomal protein L4 and/or the presence of the *cfr* ribosomal methyltransferase gene (8–11). The majority of the previous studies presenting L3 mutations have not provided evidence that the mutations are the resistance determinant, but L3 mutations have been repeatedly reported in connection with antibiotic resistance, and it is thus important to establish what effect they have.

The *rplC* gene encoding the L3 ribosomal protein is located in the S10 operon from the *str-spc* region of the *E. coli* chromosome, encoding 11 ribosomal proteins (12). The S10 operon is strongly regulated by the L4 ribosomal protein, the product of the *rplD* gene placed just downstream of the *rplC* gene (13, 14). Ribosomal proteins L3, L2, and L4 are essential and indispensable components for formation of the PTC (15, 16). These proteins are presumed to modulate peptidyl transferase activity by stabilizing and maintaining the conformation of rRNA at the active site (17). Ribosome reconstitution in *E. coli* has demonstrated that L3 is one of the first ribosomal proteins to be assembled onto the 23S rRNA and that only L3 and L24 are capable of initiating assembly of the *E. coli* 50S ribosomal subunit (18, 19). Knowledge of the effect of L3 mutations in prokaryotes is limited, but various phenotypic effects of L3 mutations have been identified in *Saccharomyces cerevisiae*. Mutations in yeast equivalent to bacterial L3 mutations affect peptidyl transferase activity, change frame-shifting efficiency, thereby altering translational fidelity, and cause rapid loss of the M1 killer virus (20). Mutations in L3 have also been shown to affect the binding of both A-site and P-site tRNA by altering the conformation of the PTC nucleotides (21) and have been shown to be responsible for trichodermin resistance (22). These observations in yeast support the notion that mutations in bacterial L3 can provide significant phenotypic effects.

In this study, we investigated 10 L3 mutations in *E. coli* that some-

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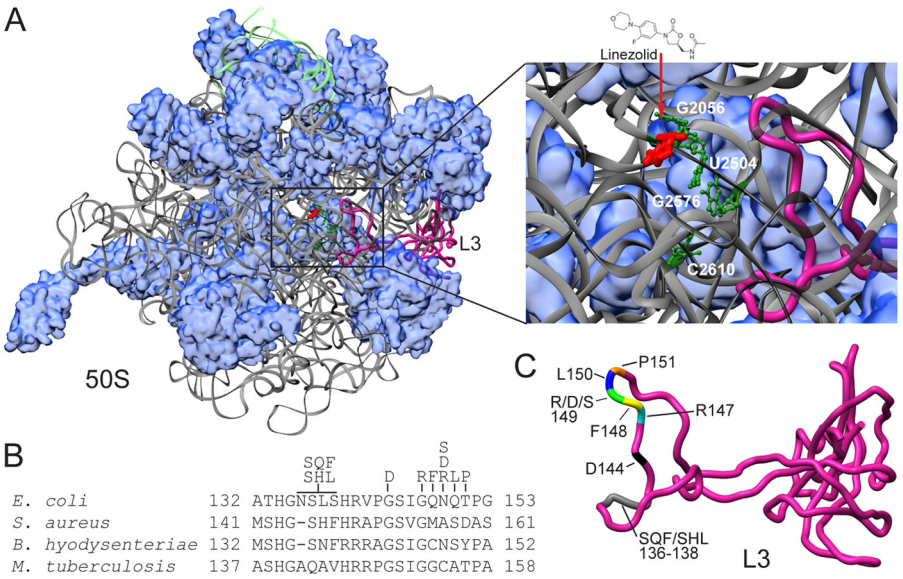

FIG 1 The ribosomal protein L3 mutations in the 50S ribosomal subunit. (A) An X-ray structure of the *E. coli* 50S ribosomal subunit (PDB file 3R8T, now superseded by 4V9D), looking down the peptide exit tunnel (open circle in the middle) with 5S RNA in light green on the top. 23S RNA is shown as gray tubing and r-proteins in light blue, except L3, which is shown as violet. At the right is a closeup of part of the PTC with linezolid (red) bound. Nucleotides G2056, U2504, G2576, and C2610, shown in green, have been calculated to exhibit structural changes upon L3 mutations. (B) Amino acid sequence alignment of a partial fragment of L3 ribosomal proteins of *E. coli*, *S. aureus*, *Brachyspira hyodysenteriae*, and *M. tuberculosis* with the mutations from this study shown above the alignment. (C) Representation of the *E. coli* L3 backbone in tubing marked with the positions of the mutated amino acids.

how relate to published studies of corresponding changes associated with antibiotic resistance to linezolid and tiamulin. The investigation of the L3 mutations was done by construction of an L3 knockout *E. coli* AS19 strain with the essential L3 gene provided on plasmids. The mutations were constructed in plasmid-carried L3 genes, and the L3-mutated plasmids were introduced via plasmid exchange into the *E. coli* AS19 L3-minus strain, replacing the L3 wild-type (wt) plasmids by antibiotic selection. We measured the doubling time of all mutant strains to assay the effect of the mutations on growth. Also, we made a computational analysis of the impact of L3 mutations on the structure of the 23S rRNA in the *E. coli* 50S ribosomal subunit, including how they affect the positioning of key nucleotides in the PTC and the binding of linezolid and tiamulin. Finally, the MICs for linezolid and tiamulin were determined for each strain and compared to those determined for the wild type to establish the effect of L3 mutations on antibiotic susceptibility.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* TOP10 strain (Invitrogen, Carlsbad, CA, USA) was used for transformation of ligated plasmids. The hyperpermeable *E. coli* AS19 strain (23) was used for gene knockout followed by MIC analysis because it is much more susceptible to antibiotics, such as tiamulin, linezolid, and other PTC-targeting antibiotics, than are other *E. coli* strains. *E. coli* AS19ΔL3 with plasmids encoding ampicillin resistance (Amp^r) was grown in LB medium with 100 μg/ml ampicillin and 40 μg/ml kanamycin. *E. coli* AS19ΔL3 with plasmids encoding tetracycline resistance (Tet^r) was grown in LB medium with 5 μg/ml tetracycline and 5 μg/ml kanamycin.

Construction of *E. coli* AS19ΔL3. A ribosomal protein L3 knockout *E. coli* AS19 strain was constructed to assess the effect of plasmid-carried L3 without a wild-type L3 background. A kanamycin resistance cassette replaced the L3 gene in the genomic S10 operon similarly to the method described by Datsenko and Wanner (24). The essential L3 was provided by induction of a plasmid-carried L3 gene on pBAD33-L3wt where the L3 gene was placed on plasmid pBAD33 (25) under the control of the arabinose-inducible promoter. The PCR product for insertion of the kanamycin resistance cassette was made by Phusion DNA polymerase with the BV389krcF and BV390krcR oligonucleotide primers (Table 1) using pKD4 (24) as the template and transformed by electroporation into AS19/pBAD33-L3wt/pKD46. Colonies were selected on agar plates containing 30 μg/ml kanamycin, 30 μg/ml chloramphenicol, and 0.2% arabinose and incubated at 37°C for 48 h. The authenticity of the genome replacement was confirmed by PCR and sequencing. pBAD33-L3wt was later exchanged with the pBR322 (26) derivatives pBR322L3WT and pBR322AmpL3 (see below) where the L3 gene had replaced the genes encoding ampicillin and tetracycline resistance, respectively. Agar plates for selection of the tetracycline-resistant plasmids contained 5 μg/ml tetracycline and 5 μg/ml kanamycin, and those for selection of the ampicillin-resistant plasmids contained 30 μg/ml ampicillin and 5 μg/ml kanamycin. The L3 expression in pBAD33-L3wt is arabinose inducible, and as the pBAD33 and pBR322 derivatives contain the same origin of replication and thus cannot stably coexist in the cell, the pBAD33-L3wt can be selected away. The exchange with pBR322L3WT was accomplished after incubation at 37°C for 16 to 18 h, while the exchange with pBR322AmpL3 additionally required incubation at room temperature for approximately 72 h. The absence of cells with pBAD33-L3wt plasmids was verified by growth on agar plates supplemented with 20 μg/ml chloramphenicol, 5 μg/ml kanamycin, and 0.2% arabinose.

Construction of pBR322 derivatives. For construction of pBR322 derivatives pBR322L3WT and pBR322AmpL3, the L3 gene was amplified from the pBAD33-L3 vector with forward primer BV433 and reverse primer BV434 (Table 1). The BV433 forward primer hybridized at the start codon of the *rplC* gene and contained an AvrII restriction site immediately upstream of the start codon. The BV434 reverse primer hybridized at the stop codon of the *rplC* gene and contained an XhoI restriction site immediately downstream of the stop codon, giving an L3 gene flanked by sequences with restriction sites for AvrII and XhoI, respectively. Addi-

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TABLE 1 DNA oligonucleotide primers[a]

Name	Sequence (5′ to 3′)	Application
BV389krcF	CTGGGTTAATCAGGTCATTGAGCGATTGAGAGG TTGAAACAATGATTGAACAAGATGGATTG	Construction of *E. coli* AS19ΔL3
BV390krcR	CTGCGCGTCTTTCAATACTAATTCCATTGCTAT CTCCTTAGAAGAACTCGTCAAGAAGGCG	Construction of *E. coli* AS19ΔL3
BV433	GGTTCCTAGGATGATTGGTTTAGTCGGT	pBR322L3WT and site-directed mutagenesis
BV434	TTAACTCGAGTTACGCCTTCACAGCTGG	pBR322L3WT and site-directed mutagenesis
BV429*bla*F	TCATCCTAGGCCTTTTTCAATATTATTG	pBR322, excluding *bla*
BV430*bla*R	GTAACTCGAGGACCAAGTTTACTCATAT	pBR322, excluding *bla*
BV431*tet*F	TATACTCGAGGCCGGCGGCACCTCGCTA	pBR322, excluding *tet*
BV432*tet*R	TCATCCTAGGTGCCTGACTGCGTTAGCA	pBR322, excluding *tet*
BV450	GCAAACAAACCACCGCTGGTAG	Sequencing the L3 gene
BV530	ACGAAAGGGCCTCGTGATAC	Sequencing the L3 gene
BV366G144DF	GGTCCGGATTCTATCGGTCAGAACCAG	Mutagenesis
BV367G144DR	CTGACCGATAGAATCCGGAACGCGGTG	Mutagenesis
BV364G147RF	TCTATCCGTCAGAACCAGACTCCGGGC	Mutagenesis
BV365G147RR	AGTCTGGTTCTGACGGATAGAACCCGG	Mutagenesis
BV437Q148FF	GGTTCTATCGGTTTCAACCAGACTCCGGGC	Mutagenesis
BV438Q148FR	CGGAGTCTGGTTGAAACCGATAGAACCCGG	Mutagenesis
BV358N149SF	TCTATCGGTCAGAGCCAGACTCCGGGC	Mutagenesis
BV359N149SR	GCCCGGAGTCTGGCTCTGACCGATAGA	Mutagenesis
BV435N149RF	TCTATCGGTCAGAGACAGACTCCGGGCAAA	Mutagenesis
BV436N149RR	GCCCGGAGTCTGTCTCTGACCGATAGAACC	Mutagenesis
BV360Q150LF	ATCGGTCAGAACCTGACTCCGGGCAAA	Mutagenesis
BV361Q150LR	GCCCGGAGTCAGGTTCTGACCGATAGA	Mutagenesis
BV362T151PF	GGTCAGAACCAGCCTCCGGGCAAAGTG	Mutagenesis
BV363T151PR	GCCCGGAGGCTGGTTCTGACCGATAGA	Mutagenesis
BV526136SQF138F	ACTCACGGTTCCCAGTTTCACCGCGTTCCGGGT	Mutagenesis
BV527136SQF138R	CGGAACGCGGTGAAACTGGGAACCGTGAGTAGC	Mutagenesis
BV528136SHL138F	ACTCACGGTTCCCATCTGCACCGCGTTCCGGGT	Mutagenesis
BV529136SHL138R	CGGAACGCGGTGCAGATGGGAACCGTGAGTAGC	Mutagenesis

[a] The mutated nucleotides in the primers used for site-directed mutagenesis are underlined; for primers BV433, BV434, BV429, BV430, BV431, and BV432, the underlined nucleotides correspond to the restriction sites for endonucleases AvrII and XhoI.

tionally, the vector fragment from pBR322 was amplified by PCR without the *bla* and *tet* genes, respectively. Primer BV429*bla*F with an AvrII restriction site and primer BV430*bla*R with an XhoI restriction site were used to amplify the pBR322 vector excluding the *bla* gene, while primer BV431*tet*F with an XhoI restriction site and primer BV432*tet*R with an AvrII restriction site were used for the amplification of the pBR322 vector excluding the *tet* gene. The L3 product and the two linear pBR322 plasmids were digested with AvrII and XhoI followed by ligation and transformation into the *E. coli* TOP10 strain.

Construction of L3-mutated plasmids. The Q148F, N149R, 136SQF138, and 136SHL138 changes were introduced into the L3 gene on pBR322L3WT using standard overlap extension PCR (27). The L3 mutated genes with G144D, G147R, N149S, Q150L, and T151P changes were introduced in a similar way into the L3 gene on pBAD33-L3wt as described above. Forward primers and reverse primers were used in combination with reverse and forward mutagenic primers, respectively, for each introduced mutation, and all primers are listed in Table 1. All mutations were finally transferred into the pBR322L3WT vector, by PCR addition of AvrII and XhoI restriction sites at the ends of the L3 gene, followed by restriction digestion of vector and fragments plus ligation, resulting in the following plasmids: pBR322L3G144D, pBR322L3G147R, pBR322L3Q148F, pBR322L3N149S, pBR322L3N149R, pBR322L3Q150L, pBR322L3T151P, pBR322L3136SQF138, and pBR322L3136SHL138. The plasmids were transformed into *E. coli* TOP10 cells and purified and finally introduced into *E. coli* AS19ΔL3 by exchange as described below. The L3 genes on the plasmids were sequenced with primers BV450 and BV530 (Table 1) to verify the presence of the mutations.

Exchange of L3-mutated plasmids into the *E. coli* AS19ΔL3 strain by antibiotic selection. pBR322 derivatives encoding tetracycline resistance and possessing either wild-type L3 or mutated L3 genes were transformed into *E. coli* AS19ΔL3 with pBR322AmpL3 plasmids encoding wild-type L3 and ampicillin resistance. Aliquots were plated on agar plates containing 5 μg/ml of tetracycline and 5 μg/ml of kanamycin and incubated at 37°C overnight. Single colonies were purified on agar plates containing identical amounts of antibiotics and incubated at 37°C overnight. The success of the replacement by antibiotic selection was verified by transfer of single colonies to agar plates supplemented with 100 μg/ml ampicillin and 40 μg/ml kanamycin to check for the absence of the pBR322AmpL3 plasmid.

Antibiotic susceptibility testing. Drug susceptibility testing was carried out using 96-well microtiter plates and measuring optical density values at 450 nm (OD_{450}) with a Victor 3 spectrophotometer (Perkin-Elmer) as described previously (28). Overnight cultures of strains in LB medium were diluted to an OD_{450} value of 0.01, and 100 μl was mixed with 100 μl of antibiotic solutions in a series with 2-fold dilution steps. Two-fold serial dilutions of the following antimicrobial agents were tested: linezolid (0.25 to 32 μg/ml), tiamulin (0.6125 to 8 μg/ml), and valnemulin (0.015 μg/ml to 2 μg/ml). The MIC was defined as the lowest concentration of antibiotic at which growth of the cultures was absent after 24 h of incubation at 37°C.

Expression of L3 by Western blotting and verification by standard mass spectrometry. The expression of the L3 protein in the L3 wild-type and L3 mutant strains was examined by Western blot analysis of *E. coli* AS19ΔL3 cells harboring the respective plasmids. Culture samples were grown under aerobic conditions and harvested at an OD_{450} of 0.75. Sam-

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TABLE 2 Growth rates, antibiotic susceptibilities, and binding energies of *E. coli* AS19ΔL3 strains[a]

Plasmid	Doubling time (min)	MIC (μg/ml)		Glide score (kcal/mol)	
		LZD	TIA	LZD	TIA
pBR322L3WT	24	8	1	−6.1	−8.6
pBR322L3G144D	130	ND[b]	ND	−4.9	−7.1
pBR322L3G147R	37	4	4	−6.2	−7.7
pBR322L3Q148F	28	8	2	−4.9	−9.1
pBR322L3N149S	27	8	8	−5.0	−7.4
pBR322L3N149D	27	4	8	−5.7	−7.2
pBR322L3N149R	29	32	1	−5.0	−7.5
pBR322L3Q150L	32	2	8	−5.1	−6.8
pBR322L3T151P	31	4	4	−5.6	−8.7
pBR322L3136SQF138	25	4	1	−4.9	−8.8
pBR322L3136SHL138	25	4	1	−5.1	−8.5

[a] LZD, linezolid; TIA, tiamulin. The L3 mutant strain with G144D was eliminated from the MIC analysis because of the large doubling time. Control strain *E. coli* AS19/pBR322 has a doubling time of 23 min, LZD MIC at 8 μg/ml, and TIA MIC at 1 μg/ml.
[b] ND, not determined.

ples were dissolved in 1× SDS–dithiothreitol (DTT) loading buffer, boiled for 5 min, and electrophoresed and separated on 12% SDS-PAGE gels along with standard markers. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Rabbit anti-L3 primary antibody (Genscript), raised against GAKKANRVTKPE AGC, was diluted to a concentration of 0.86 μg/ml, and rabbit anti-GroEL primary antibody (Enzo) was diluted to a concentration of 0.1 μg/ml. Goat anti-rabbit IgG (H&L) horseradish peroxidase (HRP)-conjugated secondary antibody (Genscript) was diluted to a concentration of 0.1 μg/ml for the GroEL and 0.3 μg/ml for the L3. Detection was done using enhanced chemiluminescence (ECL) Western HRP substrate (Millipore Corporation), followed by exposure and development on X-ray film.

Verification of the specificity of the detected band as L3 was performed by standard mass spectrometry. L3 expression in AS19ΔL3/pBR322L3WT was confirmed by excision of a band from an SDS-PAGE experiment equivalent to the band detected by Western blotting and around a size of 22 to 23 kDa according to a marker. The proteins in the SDS-PAGE piece were digested with trypsin, and the resulting spectrum was analyzed using peptide mass fingerprinting, which confirmed the L3 identity.

Determination of strain growth rates. Broth cultures of *E. coli* AS19ΔL3 possessing the wild-type L3 and mutant L3 plasmids, listed in Table 2, were diluted into fresh LB medium to an OD_{450} of 0.005. The growth rates in the exponential-growth phase were determined by growing the strains in 50 ml LB medium at 37°C under conditions of constant shaking and recording OD_{450} values every 30 min for 7 to 8 h. The data were plotted in a semilogarithmic chart, exponential regression analysis was performed on the part showing exponential growth, doubling times were calculated from the growth rates, and data represent averages of the results of two independent experiments.

Computational modeling. For the computational modeling of the effects of the L3 mutations on the 23S rRNA, an 85-Å sphere of atoms was constructed from the *E. coli* 50S ribosome structure (PDB file 3OFC, now superseded by 4V7T), centered at chloramphenicol bound in the X-ray structure. This sphere was then prepared using the protein preparation wizard in the Schrödinger Suite (Schrödinger Release 2013-3; Schrödinger Suite 2013 Protein Preparation Wizard) (Epik version 2.6, Schrödinger, LLC, New York, NY, 2013; Impact version 6.1, Schrödinger, LLC, New York, NY, 2013; Prime version 3.3, Schrödinger, LLC, New York, NY, 2013). (i) Atom types were assigned and hydrogen atoms were added followed by minimization using the OPLS2005 force field; (ii) zinc and magnesium ions were kept in the model, while chloramphenicol and water were removed; and (iii) protonation and tautomer/flip states were determined for the protein part of the system. To take into account effects of the solvent (although the effects were small due to the large size of the system sphere), the Desmond program (Schrödinger Release 2013-3) (Desmond Molecular Dynamics System v, D. E. Shaw Research, New York, NY, 2013; Maestro-Desmond Interoperability Tools, version 3.6, Schrödinger, New York, NY, 2013) was used to set up an orthorhombic solvation box with a 10-Å buffer around the PTC model. The simulation box contained approximately 575,000 water molecules, 2,000 Na^+ ions for neutralizing the model, and 150 mM NaCl (approximately 550 molecules). Energy minimization was accomplished in a stepwise fashion, and all atoms were minimized to a 0.5 kcal/mol convergence for the total energy in the last minimization round.

The different L3 mutations were made using the automated procedure implemented in Schrödinger followed by energy minimization: first by energy minimization in the local region around the mutated residue (including all residues within 6 Å of the mutated amino acid) and then by complete energy minimization. The root mean square deviation (RMSD) calculations for selected residues were calculated with the "rmsd_by_residue.py" script from Schrödinger's script database (version 2013-3). The RMSD reference model was set up in the same way as for the mutated PTC models (described above) but with the wild type. All water molecules and sodium and chloride ions were removed from the minimized systems. All the docking calculations were performed using the Glide program in XP (extraprecision) mode (Small-Molecule Drug Discovery Suite 2014-3) (Glide v, Schrödinger, LLC, New York, NY, 2014). The 85-Å sphere described above was reduced to a 60-Å sphere because of limitations with respect to the size of the system that can be considered in the Glide program and was then used for docking. The Van der Waals radius scaling factor for the nonpolar atoms in the ligand was set to 0.9, and the corresponding factor for the receptor atoms was set to 0.8. A scaling factor of less than 1 is usually used in order to simulate receptor flexibility. Finally, the partial-charge cutoff values were 0.15 for the ligand and 0.25 for the PTC model.

RESULTS

Construction of an *E. coli* L3 knockout strain with plasmid-encoded L3 protein and replacement of wild-type L3 gene with mutant L3 genes via plasmid exchange. As L3 ribosomal protein (uL3 according to the new universal naming of ribosomal proteins [29]) is essential for the viability of *E. coli* and as we sought to investigate mutated L3 relative to the wild type, we constructed an *E. coli* strain with an *rplC* genome knockout supplemented with a plasmid-carried L3 gene expressing the essential L3 protein. The hyperpermeable *E. coli* AS19 strain was used to monitor the effect of the mutations as this strain is particularly sensitive to many antibiotics (23). The L3 gene (*rplC*) in the genomic S10 operon was replaced by a kanamycin resistance cassette as described by Datsenko and Wanner (24), while the essential L3 gene was supplied on a pBAD33 derivative (pBAD33-L3wt) under the control of an arabinose-inducible promoter. Subsequently, we wanted constitutively expressed ribosomal L3 protein, and pBAD33-L3wt was therefore replaced by pBR322 derivatives. In the pBR322 derivatives, the ampicillin resistance gene (*bla*) and the tetracycline resistance gene (*tet*) were replaced with the L3 gene, forming plasmids pBR322L3WT (Tet^r) and pBR322AmpL3 (Amp^r), respectively. The L3 genes were positioned so that they used the promoters of the genes that they replaced and were thus constitutively expressed.

A plasmid exchange system was developed to express mutated L3 in the *E. coli* AS19ΔL3 strain by exchange of plasmids with wild-type L3 genes with those encoding mutated L3. The exchanged plasmids are derivatives of pBR322 and contain the same origin of replication and thus cannot stably coexist in the bacteria (30). pBR322L3WT (Tet^r) and pBR322AmpL3 (Amp^r) were utilized to introduce mutated L3 genes into the *E. coli* AS19ΔL3

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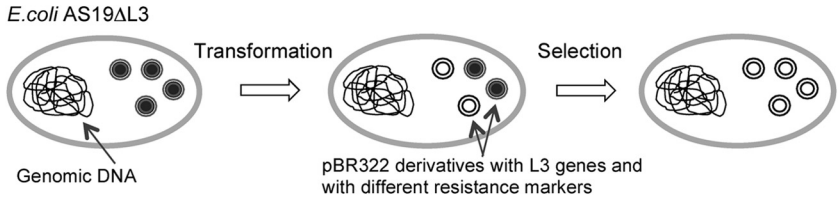

FIG 2 A schematic illustration of the exchange procedure replacing plasmid-carried wild-type L3 with plasmid-carried mutated L3. Filled circles illustrate pBR322AmpL3 (Amp^r) and open circles pBR322mutatedL3 (Tet^r). In the case shown, the selection was performed with tetracycline.

strain by swapping. As illustrated in Fig. 2, L3-mutated plasmids with tetracycline resistance were transformed into *E. coli* AS19ΔL3 with a wild-type L3 provided on pBR322AmpL3, encoding ampicillin resistance. Exposing the cells to tetracycline creates a selection for *E. coli* AS19ΔL3 cells with only L3-mutated plasmids and, subsequently, ribosomes with only mutated L3. The substitution of L3 wild-type plasmids with L3-mutated plasmids was verified by the absence of colonies by plating on agar plates containing ampicillin. The exchange was attempted using the antibiotics in the opposite direction. Replacement of plasmids encoding tetracycline resistance with plasmids encoding ampicillin resistance failed in several attempts for unknown reasons. This issue was previously observed and investigated (30).

Selection of specific mutations for introduction into ribosomal protein L3 in *E. coli*. Mutations in ribosomal protein L3 near the PTC have been associated with resistance to linezolid and tiamulin in several important clinical pathogens. In order to analyze what role these amino acid mutations play in antibiotic resistance or otherwise, we selected six L3 mutations from literature studies: G144D (31), G147R (5, 6, 32) N149S (33), N149D (4), N149R (7, 34), and Q150L (5, 6) (*E. coli* numbering). These mutations have been discovered in either tiamulin-resistant or linezolid-resistant strains without any other known resistance determinants. Some of them have been revealed to be directly involved in the resistance, while the effect remains to be elucidated for others as discussed below. In addition, L3 mutations in the region corresponding to positions 136 to 139 of *E. coli* L3 have been found in 23 *S. epidermidis* isolates with lowered susceptibility to linezolid (8, 35–37) but always together with other resistance determinants. To explore this region of L3, we made two mutants with double amino acid changes and one deletion. Finally, we introduced L3 mutations Q148F and T151P; those two mutations have not previously been identified, but other amino acid substitutions at the same position and in the proximity have been found (8, 38–41). Tables 3 and 4 list the mutations in L3 that have so far been associated with resistance to linezolid and tiamulin.

TABLE 3 Mutations in L3 that have been associated with linezolid resistance

L3 mutation(s)	*E. coli* numbering	Organism(s)	Remark(s)[a]	Reference(s)
ΔF127-H146	118–138	*S. aureus*	*In vitro*	32
Q136H/H146Δ	127/138	*S. aureus*	L4 mut	42
G137A	128	*S. epidermidis*	23S rRNA mut	36
G137S/H146R/V154L/M156T	128/138/146/148	*S. epidermidis*	23S rRNA mut, L4 mut	39
G137D/H146P/M156R	128/138/148	*S. epidermidis*	23S rRNA mut, L4 mut	39
G139R	129	*S. aureus*	T, 23S rRNA mut	31
G139R/M156T	129/148	*S. hominis*	T, 23S rRNA mut	40
ΔS145	136	*S. aureus*		43
ΔS145/H146Y	136/138	*S. aureus*	*cfr*	9
H146R/M156R	138/148	*S. epidermidis*	23S rRNA mut, L4 mut	39
H146R/M156T	138/148	*S. epidermidis*	T, 23S rRNA mut, L4 mut	8, 39
H146Q/V154L/A157R	138/146/149	*S. epidermidis*	T, L4 mut	8
H146Q	138	*S. epidermidis*	L4 mut	36
H146Q/V154L	138/146	*S. epidermidis*	23S rRNA mut, L4 mut	42
H146Q/V154L/A157R	138/146/149	*S. epidermidis*	23S rRNA mut, L4 mut	35
F147L	139	*S. epidermidis*	±*cfr*, ±L4 mut	36, 42
F147I	139	*S. epidermidis*, *S. hominis*	T, 23S rRNA mut	8, 40
F147L/A157R	139/149	*S. epidermidis*	±*cfr*, L4 mut	37
G152D	144	*S. aureus*, *S. haemolyticus*, *S. epidermidis*	±*In vitro*, ±T, ±*cfr*, ±23S rRNA mut	10, 31, 32, 44
G152D/D159Y	144/151	*S. epidermidis*	T, 23S rRNA mut	45
G152D/D159E/A160P	144/151/152	*S. epidermidis*	T, 23S rRNA mut	46
V154L/M156T	146/148	*S. epidermidis*	23S rRNA mut	39
G155R	147	*S. aureus*	*In vitro*	32
G155R/M169L	147/161	*S. aureus*	*In vitro*	32
M156T	148	*S. haemolyticus*	T, *cfr*, 23S rRNA mut	47
A157R	149	*S. epidermidis*	23S rRNA mut	43
S158Y/D159Y	150/151	*S. epidermidis*	*cfr*	41
S158F/D159Y	150/151	*S. cohnii*	*cfr*, L4 mut	41
Y158F	150	*S. cohnii*	*cfr*	10
ΔM169-G174	161–166	*S. aureus*	*cfr*	9
C154R	149	*M. tuberculosis*	±*In vitro*, ±23S rRNA mut	7, 34
H155R	150	*M. tuberculosis*		34

[a] All isolates were clinical except the ones marked "*In vitro*," which were *in vitro*-selected mutants. Selected additional information: T, treatment with linezolid; *cfr*, contained *cfr* gene. For the potential additional resistance determinants, "23S rRNA mut" refers to mutations in 23S rRNA positions and "L4 mut" indicates L4 mutations.

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TABLE 4 Mutations in L3 that have been associated with tiamulin resistance[a]

L3 mutation(s)	*E. coli* numbering	Organism	Remark(s)	Reference(s)
R141S		*E. coli*		6
G144R/G152D/D159Y	135/144/151	*S. aureus*		5
N148K	149	*B. hyodysenteriae*	23S rRNA mut	33
N148S	149	*B. hyodysenteriae*	23S rRNA mut	33, 48
N148S	149	*B. pilosicoli*	23S rRNA mut	33
R149S	141	*S. aureus*		6
N149D	149	*E. coli*		4
S149I	150	*B. hyodysenteriae*		33
S149T	150	*B. hyodysenteriae*	Animal isolates, 23S rRNA mut	48
G152V	148	*S. roseosporus*		38
G152D	144	*S. aureus*		5
G152D/G155R	144/G147	*S. aureus*		5
G152D/D159Y	144/T151	*S. aureus*		5
G152D/G155R/D159Y	144/147/151	*S. aureus*		5
G153R	153	*E. coli*		6
G155R	147	*S. aureus*		5, 6
K157Q	157	*E. coli*		6
S158L	150	*S. aureus*		5, 6
D159Y	151	*S. aureus*		5

[a] All isolates were *in vitro*-selected mutants except the ones referred to as "animal isolates." "23S rRNA mut" refers to mutations in 23S rRNA.

The 10 mutations are depicted in Fig. 1B and C and aligned with the equivalent sequences from selected species. Nine of the 10 mutations were introduced into the L3 gene on plasmids by overlap extension PCR. Concurrently, restriction sites flanking the coding regions to be used for cloning were introduced. The N149D L3 mutation was obtained from a plasmid from a previous study (4) and was transferred via PCR amplification and cloning. All 10 L3 mutated genes were cloned into the pBR322L3WT vector–a pBR322 derivative with the coding region of the *amp*^{r} gene replaced by the coding region of the L3 ribosomal protein gene and containing a *tet*^{r} gene.

Growth rates of the L3 mutant strains. In order to assess the impact of the introduced L3 mutations on the growth rate of the strains, the doubling times of all L3 mutant strains were measured under optimal conditions, and the results are presented in Table 2. The small difference in doubling time between AS19 (21 min) and AS19/pBR322 (23 min) might have been due to maintenance of the plasmid copy number and expression of the *amp*^{r} and *tet*^{r} genes. The doubling times for AS19/pBR322 and AS19ΔL3/pBR322L3WT (23 min) were identical, showing that the replacement of the chromosomal expression of the L3 gene with the plasmid-carried wild-type L3 gene did not significantly affect the growth of the bacteria under optimal conditions. An increased doubling time was observed for all L3 mutant strains, but only L3 mutations G144D and G147R resulted in severe growth reductions, with doubling times at 130 min and 37 min, respectively. The other L3 mutant strains grew relatively well, with doubling times from 25 min to 32 min. Due to its very high doubling time, AS19ΔL3/pBR322L3G144D was excluded from further laboratory investigations.

Verification of L3 expression by Western blotting and mass spectrometry. To verify expression and stability of the mutated L3 ribosomal protein, a Western blot analysis was performed to assay the presence of L3 in all strains. An antibody against a 15-amino-acid peptide present in all L3 mutants and the wild type was bound to membrane blots from SDS gels, with GroEL as a reference band (data not shown). The blot shows that mutated L3 was present in the mutants in amounts comparable to those seen with the wild-type *E. coli* AS19 strain. Together with the results from the growth analysis, the data indicate that the L3 expression levels in the mutant strains were sufficient for new ribosome assembly.

To ensure the specificity of the antibody in the Western blot analysis, standard mass spectrometry analysis was performed on the band considered to represent L3 expressed in AS19ΔL3/pBR322L3WT. A band from an SDS-PAGE experiment equivalent to the band detected by Western blotting was excised and digested with trypsin, and the resulting spectrum was analyzed using standard peptide mass fingerprinting, confirming the L3 identity.

Antibiotic susceptibilities of L3 mutant strains. The effect of the L3 mutations on antimicrobial susceptibility was investigated by MIC assays. Determinations of the MICs for linezolid and tiamulin were performed for all L3 mutant strains as well as for the wild type, except for L3 G144D, because of its long doubling time, and the results are presented in Table 2. Only the *E. coli* AS19ΔL3/pBR322L3N149R mutant strain showed a considerably higher linezolid MIC (32 μg/ml) than L3-wild-type strain *E. coli* AS19ΔL3/pBR322L3WT (8 μg/ml). The other L3 mutants showed either wild-type susceptibility or a slightly lower value not considered significantly different (less than a 2-fold difference). The L3 Q150L mutant showed higher susceptibility (2 μg/ml) to linezolid than the rest of the strains, indicating moderate hypersensitivity. Five of the nine mutants investigated showed a higher tiamulin MIC than the wild type. The highest tiamulin MICs (8 μg/ml) were observed for the L3 mutant strains with N149S, N149D, and Q150L. Slightly lowered tiamulin susceptibility results were observed for the L3 mutant strains with G147R and T151P, with a tiamulin MIC of 4 μg/ml compared to 1 μg/ml for the wild type. All five strains also exhibited 2-fold-lower susceptibility to the pleuromutilin valnemulin (from 0.06 μg/ml for the wild type to 0.12 μg/ml for the mutants; data not shown), which supports the tiamulin data. The effects for each strain are discussed further below.

Computational modeling of the effects of L3 mutations on

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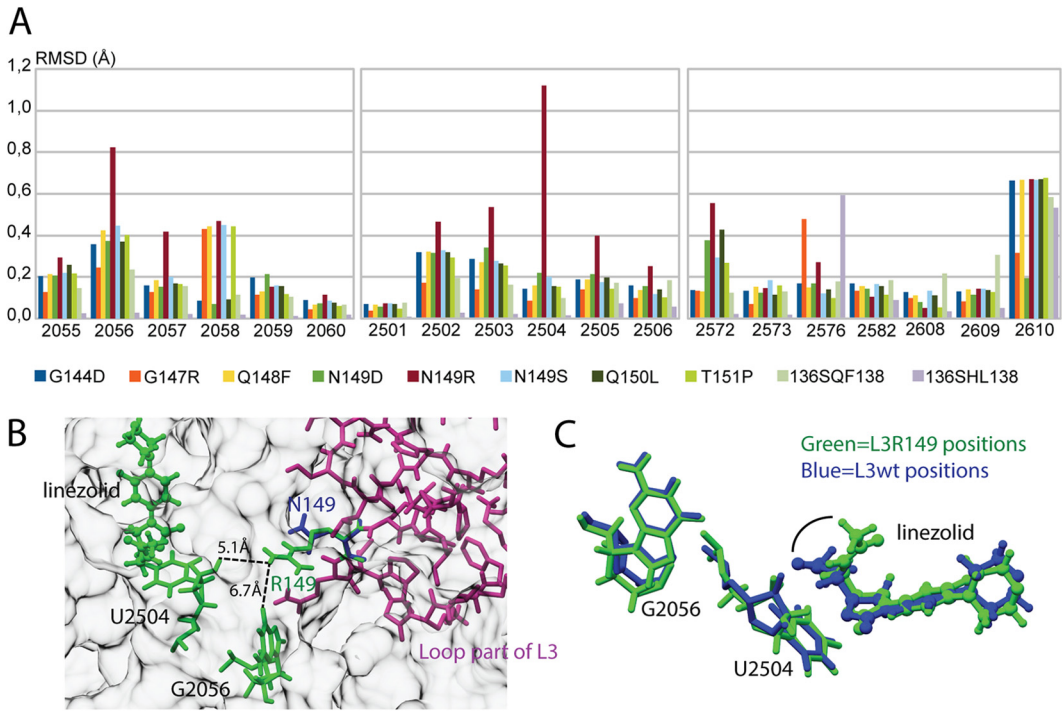

FIG 3 Illustration of the computational modulations. (A) A selection of structural changes upon mutations measured in terms of RMSD with respect to the wild type. The selected nucleotides are shown on the *x* axis with the RMSD (Å) from all L3 mutations (color code beneath the graph) on the *y* axis. (B) A closeup cutaway image of the 60-Å docking sphere from the X-ray structure of the *E. coli* 50S (PDB file 3OFC) with the L3 mutation R149 modeled into the structure. The nucleotides most affected (see panel A above) by the mutation are shown as green sticks together with the calculated position of linezolid (ball and stick). The wild-type N149 is superimposed in blue. The distances from the mutated R149 amino acid to nucleotides G2056 and U2504 are shown. (C) Superposition of U2504, G2056, and linezolid from the N149R L3 and the wild-type L3 docking structures. The curved line points to the calculated structural shift of linezolid resulting from the presence of the mutated R149 amino acid.

the structure of the 50S ribosomal subunit and antibiotic binding. As the strain knockout, plasmid cloning, and mutagenesis procedures are time-consuming, we investigated whether computational modeling of the L3 mutations could provide a useful and reliable estimation of the mutational effect on antibiotic binding and structural changes.

To investigate antibiotic binding, we used docking, employing the XP Glide methodology, which can quantify the binding mode and energy of binding of ligands to a receptor (49). This docking formalism predicts a docking score, called the glide score, that reflects how strongly a given ligand binds to the receptor, which, in our case, was binding of antibiotics to the 50S ribosomal subunit from *E. coli*. In addition to this docking score, a positioning of the ligand at the binding site is predicted. As the explicit inclusion of the whole 50S subunit in the receptor model would represent a computational challenge, and as we expected only local changes, a sphere of atoms around the antibiotic binding site was selected to represent the receptor model for the calculations. Such cluster models are extensively used within computational biology and usually represent a good compromise between accuracy and efficiency. The L3 mutations were imposed into the 50S structure, and docking experiments were performed with linezolid and tiamulin as ligands. The predicted glide scores are listed in Table 2 together with the MIC results. Due to the highly parameterized nature of such calculations, the glide score should change by at least around 1 kcal/mol to express a clearly significant effect. As seen in Table 2, the values obtained for the modulated structures docked with either linezolid or tiamulin are fairly similar to the glide scores for docking based on the wild-type structure. Thus, based solely on the docking score, no decisive conclusions can be made at this stage.

To quantify structural changes introduced by the mutations, we calculated the root mean square deviation (RMSD) values for selected nucleotides and amino acids using the wild type as the reference. The RMSD provides the average change in displacement of a selection of atoms for a particular structure, correlating it to a reference structure, which in our case is a measure of how much the atoms within a given nucleotide or amino acid have moved on average after introduction of the specific L3 mutations. 23S rRNA nucleotide positions 2055 to 2063, 2447 to 2455, 2497 to 2507, 2572 to 2573, 2576, 2582 to 2588, and 2606 to 2610 were selected for investigation as they have previously been reported to be involved in binding of tiamulin and linezolid. RMSD values that change less than 1 Å are not generally considered significant; nevertheless, we looked closely at all RMSD values above 0.6 Å, as some nucleotides clearly shifted. The most interesting results are presented in Fig. 3A. The highest RMSD values were found for nucleotides G2056 (0.82 Å), U2504 (1.12 Å), G2576 (0.60 Å), and C2610 (0.68 Å), and, as depicted in Fig. 1A, these positions are all in close proximity to the part of the L3 protein where the mutations are placed. There is thus in general a good correlation between nucleotide RMSDs and their distances to the L3 mutations. As the calculated structural changes are very small, the results

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point to the accumulation of small structural changes in the 23S rRNA leading to lower affinity for the antibiotics.

DISCUSSION

In the following, the individual L3 mutations in *E. coli* and their effects are discussed and related to appropriate results in the literature. The single-amino-acid mutations appear in numerical order to ease comparison with L3 mutations in other bacteria (Tables 3 and 4), while the two 136NSLS139 mutations are discussed at the end.

The L3 G144D mutation in *E. coli* is equivalent to *Staphylococcus* L3 G152D, which has been reported in *Staphylococcus aureus*, *S. haemolyticus*, and *S. epidermidis* as a possible resistance determinant for both linezolid and tiamulin (see Tables 3 and 4 for references). The G152D mutation appears to be the only modification in some *Staphylococcus* strains, while other strains have additional resistance determinants. For example, the triple L3 mutations G144R/G152D/D159Y and G152D/G155R/D159Y appeared in *S. aureus* under conditions of exposure to tiamulin and, for G144R/G152D/D159Y, it was verified that the increased resistance was solely due to these three L3 mutations (5). All three of the mutations G152D, G155R, and D159Y resulted in decreased susceptibility to tiamulin in *S. aureus* when found alone, but increased resistance was observed when they were found together, suggesting that coupled L3 mutations give higher resistance than single L3 mutations (5). The G144D mutation was suggested to be the only resistance determinant in a linezolid-resistant *S. aureus* isolate (31) and has been suggested to reduce oxazolidinone affinity by indirect perturbation of bases G2505 and U2506 of 23S rRNA (32). The incorporation of L3 G144D into *E. coli* caused a large growth inhibition with a doubling time of 130 min, and we therefore did not pursue the effect of this mutation further. The inhibition is surprising, as the same mutation seems to be well tolerated in *S. aureus* and is placed in a relatively well-conserved area in L3. The mutation changed the smallest amino acid glycine to the larger and charged aspartic acid, and this might have an impact on the folding or the tertiary structure, seemingly inhibiting either incorporation of L3 into ribosomal subunits or function of the 50S. The RMSD values in Fig. 3A (and data not shown) do not suggest a major structural local change from this mutation to explain this. The mutation has been reported in several unrelated studies in *Staphylococcus* spp. (5, 10, 31, 32, 44–46), and it is thus concluded that this mutation is much better accommodated in *Staphylococcus* spp. than in *E. coli*.

The L3 G147R mutation in *E. coli* is equivalent to L3 G155R in *S. aureus*, which has been related to both linezolid and tiamulin resistance (see Tables 3 and 4 for references), and was proven to be a single determinant that can confer cross-resistance to these antibiotics (6). In *E. coli*, it caused considerable growth retardation (37 min compared to 23 min for the wt) but also a small but significant rise in the tiamulin MIC (4 μg/ml compared to 1 μg/ml for the wt). Although it has previously been suggested as the only resistance determinant in a *S. aureus* strain with a 2-fold increase in linezolid resistance (32), no lowered susceptibility to linezolid was seen with G147R in *E. coli* (Table 2). The G147R mutation has been implicated in disturbing linezolid binding by disrupting U2504 (32) and was suggested to indirectly disturb the conformation of 23S RNA G2576, affecting tiamulin binding (6). As for G144D, it is a mutation that changes the small glycine to an amino acid with a larger side chain, which might impair the local tertiary structure. The RMSD values suggest a minor structural local change from this mutation on 23S RNA G2576, where mutations are known to cause linezolid resistance. Although no reduced susceptibility to linezolid resulting from L3 G147R in *E. coli* was observed, there was a clearly reduced tiamulin MIC. This report thus shows that a mutation at amino acid 147 in *E. coli* L3 can decrease tiamulin sensitivity as a single determinant.

The L3 Q148F mutation in *E. coli* has not been observed in earlier studies, but other mutations at corresponding positions in *Staphylococcus* spp. have been found. In *S. haemolyticus*, a L3 M156T mutation has been observed together with *cfr* and 23S RNA G2576T (47), which are both well-known linezolid resistance determinants. The same mutation has also been found in 13 linezolid-resistant *S. epidermidis* isolates (8, 39) and in two linezolid-resistant *S. hominis* strains (40). Also, a M156R mutation has been found in two linezolid-resistant *S. epidermidis* isolates (39). As these mutations were all found together with mutations in 23S rRNA, their contribution to linezolid resistance is unclear. Peculiarly, a G152V mutation, at the position equivalent to 148 in *E. coli*, has been found in a pleuromutilin-resistant *Streptomyces roseosporus* strain showing increased production of daptomycin (38). In our study, the L3 Q148F mutation showed no increased MIC of linezolid or tiamulin, so there are no indications that this L3 position has relevance for antibiotic resistance in *E. coli*. However, there was a growth inhibition (28 min compared to 23 min for the wt), indicating that the glutamine-to-phenylalanine change at position 148 is allowed but comes with a small cost.

L3 position N149 in *E. coli* is the point of L3 closest to the PTC, supporting the notion that changes can be directly involved in resistance to binding of antibiotics in this region. The effects of three single mutations at L3 position N149 in *E. coli* were investigated because the mutations have been found in various bacteria but also to investigate the effect of different amino acid changes at the same position in relation to antibiotic resistance. The L3 N149D in *E. coli* was the first of the bacterial L3 mutations to be related to antibiotic resistance (4) but resulted in a very moderate increase in the tiamulin MIC. The *E. coli* L3 N149R equivalent in *S. epidermis*, A157R, has been found together with 23S RNA G2447T, which provides linezolid resistance on its own (43). A157R has also been found in other linezolid-resistant *S. epidermidis* strains together with other L3 and L4 mutations and 23S RNA C2534T (8, 35). In *Mycobacterium tuberculosis*, the corresponding L3 C154R mutation provides linezolid resistance (7, 34). Finally, the *E. coli* N149S mutation is equivalent to the L3 N148S mutation observed in two *Brachyspira* species (33, 48) with a possible link to tiamulin resistance.

The growth inhibition (27 to 29 min compared to 23 min for the wt) for all three N149 mutations in *E. coli* (Table 2) shows that the changes are allowed but come with a small cost. 149D and 149S increased the tiamulin MICs from 1 μg/ml to 8 μg/ml, and 149R increased the linezolid MICs from 8 μg/ml to 32 μg/ml (Table 2). Effects seen in one species might not be equivalent to similar changes in other species, but it is known from 23S RNA mutations that this is often the case. Also, it is striking that the *E. coli* L3 N149R mutation that decreased linezolid sensitivity is the same amino acid change as the one related to linezolid resistance in both *S. epidermidis* and *M. tuberculosis* as mentioned above. The same goes for the 149S mutation that decreased tiamulin sensitivity in *E. coli* and has been related to tiamulin resistance in *Brachyspira hyodysenteriae*. Also, the fact that N149S and N149D affect suscepti-

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bility to tiamulin but not susceptibility to linezolid whereas N149R affects susceptibility to linezolid but not susceptibility to tiamulin underscores the specificity of the effects of the mutations.

The correlation between MIC values and docking scores (Table 2) for the amino acid 149 mutations is not impressive but may reflect the possibility that even small changes in binding energies can affect the MICs. The RMSD values for N149R show a significant effect on 23S RNA nucleotide U2504 (Fig. 3A), suggesting a structural effect in this area. Figures 3B and C depict the computational modulation of the L3 N149R mutation in the presence of linezolid, where the mutation changes the position of the phosphate group at U2504 and shows a slight effect on G2056, probably provided by the positive charge on the arginine. The structure shows the close proximity between L3 149R, U2504, and linezolid and subtle changes in conformations of U2504 and linezolid relative to the wild-type structure. The model supports the notion that single amino acid changes in this area of L3 can cause small local changes in nearby nucleotides that perturb the antibiotic binding. Although small, the RMSD values for nucleotides U2506 and C2610 are also consistent with this view, as they are placed very close to the antibiotic binding site as seen in Fig. 1.

L3 Q150 in *E. coli* corresponds to L3 S158 in *S. aureus*, where a S158L mutation has been found to give reduced susceptibility to tiamulin (5, 6). The resistance genotype was rationalized by the mutation causing a perturbation of the conformation of U2504 as U2504 is a key residue for tiamulin binding (6). In our study, the *E. coli* L3 Q150L mutation confers resistance to tiamulin with an 8-fold increase from 1 μg/ml to 8 μg/ml (Table 2). We can thus confirm the previous data and suggest that the L3 Q150L mutation is not species specific but may be found in various species. We find an extended doubling time from 23 min for the wt to 32 min (Table 2), again suggesting that the mutations come with a cost. According to the RMSD data, the mutation does not seem to affect U2504 or other 23S rRNA nucleotides significantly and the only and maybe significant change in RSMD is seen for C2610.

A D159Y mutation, corresponding to position 151 in *E. coli*, has been found in two *S. epidermidis* isolates together with *cfr* and L3 mutation S158Y and has been found in one *S. cohnii* isolate together with *cfr*, L3 mutation S158F, and additional L4 mutations, all exhibiting elevated linezolid MICs (32 μg/ml) (41). It has been suggested by Mendes et al. that L3 mutations S158Y and D159Y act synergistically with *cfr* and somehow contribute to the elevated linezolid MIC results (41). Also, in another study, five *S. epidermidis* isolates with linezolid MICs of >256 μg/ml harbored the D159Y but also 23S rRNA mutations and other L3 mutations (45), and these other mutations might be responsible for the elevated MICs. The D159Y mutation has also been found in *S. aureus* isolates with reduced susceptibility to tiamulin, and these strains exhibited a slight defect in growth (5). In our study, the L3 T151P mutation in *E. coli* at the position corresponding to L3 position 159 in *Staphylococcus* spp. resulted in a reduction of the susceptibility to tiamulin, with a 4-fold increase in the MIC from 1 μg/ml to 4 μg/ml, but no change in sensitivity to linezolid (Table 2). This is in accordance with the tiamulin effect described above, but we cannot support any conjecture of an influence on linezolid MICs from this mutation. As also reported for *S. aureus* (5), we found an extended doubling time, from 23 min for the wt to 31 min (Table 2), similar to what is seen for the neighboring Q150L mutation discussed above.

A number of mutations and a deletion have been found in L3 at position 145SHF147 in *S. epidermidis* and *S. aureus* strains with reduced susceptibility to linezolid (see Table 3 for details on mutations and references). Except for one, they are all found together with other L3 mutations, L4 mutations, 23S RNA mutations, or the *cfr* gene. The exception is a ΔS145 mutation from a clinical *S. aureus* isolate with no report of other known resistance determinants (43). The *Staphylococcus* L3 145SHF147 region corresponds to 136NSLS139 in *E. coli* L3, which is thus one amino acid longer. To investigate this region, we made two mutants, 136SQF138 and 136SHL138, each with both a deletion and two mutations, to make mimics of the *Staphylococcus* region. L3 mutations 136SQF138 and 136SHL138 in *E. coli* showed only a small effect on doubling times (25 min compared to 23 min for the wt), indicating that these mutations are well tolerated. They showed no effect on either linezolid or tiamulin susceptibility, as seen in Table 2. Also, the X-ray structure shows that the L3 136NSLS139 branched-loop area lies >20 Å away from the linezolid binding site. There is thus no indication in our *E. coli* study to support a contribution from L3 mutations in this area to the observed resistance in *Staphylococcus* spp. We cannot exclude the possibility of such a contribution, but find it more likely that these L3 mutations play some other role. They might somehow facilitate the presence of the resistance determinants with which they are found. For example, the linezolid resistance caused by a mutation at position 2576 in 23S RNA has been shown to confer a growth defect in *S. pneumoniae*, but this fitness cost was rescued by a L3 mutation at position Y137H (50). This Y137H mutation, which did not confer any reduced susceptibility to linezolid alone, is at the L3 position in *S. pneumoniae* that corresponds to the *E. coli* L3 S139.

Conclusion. We have constructed an *E. coli* AS19ΔL3 strain and developed a plasmid exchange system to replace plasmid-carried wild-type L3 genes with mutant L3 genes. This enabled us to express mutated L3 genes on plasmids in the *E. coli* AS19ΔL3 strain and thus to investigate the effect of L3 mutations without any selective pressure under conditions comparable to those corresponding to similar wild-type expression. Of the 10 investigated mutants, five exhibited decreased sensitivity to tiamulin and one showed decreased sensitivity to linezolid. Three mutations did not show an effect on antibiotic resistance, although mutations at corresponding positions in L3 genes in other bacteria have been related to antibiotic resistance. This is not surprising for at least three reasons. (i) As the L3 genes from various bacteria are not very well conserved along the whole gene, it cannot be expected that a mutation in a specific area would exert the same effect in different bacteria. (ii) Many of the reported L3 mutations are found together with the well-known resistance determinants, namely, 23S rRNA mutations and the *cfr* gene, and even though they might appear for a reason, they do not necessarily have to contribute on their own to resistance. In addition, many of the strains harbor several L3 and L4 mutations that may or may not be individually implicated to resistance. (iii) It is likely that some of the L3 mutations that arise in antibiotic-resistant strains rescue some detrimental effects of the other resistance determinants, as in the example presented in reference 50. Alternatively, they might provide some growth advantages under certain conditions by helping the bacteria to survive and multiply and thereby provide the opportunity to obtain antibiotic resistance by some other mechanism.

We also explored whether computational modeling could demonstrate the effect of L3 mutations on antibiotic binding and

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on the structure of the binding pocket. If computer calculations can make reliable predictions about mutations, this could indeed represent a valuable tool as a supplement to the laboratory work. The docking scores point to only small changes in the binding energies of linezolid and tiamulin, and the span in these binding energies is too small to make any decisive conclusions. The MIC changes are also moderate, so we cannot conclude whether such calculations might be useful. The RMSD values show very moderate changes of the structure, but the nucleotides predicted to be influenced are in fact those located close to the L3 mutations and might thus reflect the actual changes. Also, no dramatic structural effects are expected, as those would probably not be allowed in such an important and well-conserved region as the peptidyl transferase area.

Our "knockout plus plasmid" model has convincingly shown that six of nine investigated L3 mutations affect antibiotic function in *E. coli* and that many of the mutations are relatively well tolerated. The literature with L3 mutations point to the fact that they are often present together with other possible resistance determinants, so there is still a big challenge in determining the cooperative effect of multiple players in antibiotic resistance.

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